ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM

(U01 DK61018)

UPDATE REPORT
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Table of Contents

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PART A:

PRINCIPAL INVESTIGATOR'S SUMMARY

Program Accomplishments:

The major theme of the AMDCC project at Vanderbilt is the identification and characterization of genetic modifiers causing diabetic nephropathy. Within this theme three related topics of research are: 1) characterization of candidate gene mutations exacerbating nephropathy in mice; 2) characterization of ENU mutants exhibiting diabetic nephropathy, and 3) Establishment of Phenotyping capability for renal function in diverse strains of mice.

Major accomplishments include:

1) Determination that eNOS-/- db/db mice exhibit more severe diabetic nephropathy than ApoE, LDLR or Cyp4a14 knockout mice. This model looks is the most robust model of DN we have characterized thus far, exhibiting both decreased GFR and robust albuminuria.
2) Further characterization of five novel ENU mutants that exhibit excess albuminuria in the setting of diabetes as compared with other mice of the same strain. Some of these lines appear to have decreased GFR.
3 We are determining the role correlation between FBS, HbA1C, glycated Hb in several mouse strains.
4) We are pursing the characterization of mouse strains that exhibit more severe albuminuria and histopathological changes than C57BL/6J, especially KK and DBA.

Interrelationship of projects:

Project 1: Project 1 – " Characterization of Candidate genes predisposing to diabetic nephropathy.

This project is focused primarily on type II diabetes and defining the role of dyslipidemia and hypertension in exacerbating diabetic nephropathy. Project 2 is characterizing type I diabetes. Both are utilizing phenotyping techniques validated to measure GFR, albuminuria, and glomerular histopathology in mice that are being developed by project 3.

Project 2

In collaboration with Dr. Gene Rinchik a member of our consortium at University of Tennessee, in a project directed by Dr. Breyer, we have generated over 300 diabetic ENU mutants and identified five mutants with increased albuminuria. Drs Rinchik and Breyer, will increasingly collaborate as we initiate a mapping strategy for these mutants. Drs. Breyer, and Fogo have collaborated in characterization of renal pathological changes occurring in diabetic strains.

Project 3

Project 3 involves close work with projects 1 and 2 and has evolved a major goal of phenotyping diabetes and diabetic nephropathy in mice. Within the AMDCC, This year, Dr. Zhonghua Qi in Dr. Breyer's group has collaborated with Drs. Fogo and Harris to develop techniques to measure GFR in mice. Dr. Qi is also characterizing renal function

in several strains of mice. Results from project 3 are critical for defining benchmarks for normal vs. abnormal renal function, fasting glucose, HbA1C, GFR, albuminuria and renal histopathologic criteria. Among the important results from this project will be identification of mouse strains susceptible and resistant to diabetic nephropathy. Mapping of dominant ENU generated mutations predisposing to DN in C57BL/6J mice will depend on the identification of another strain (other than C57BL/6J) that is resistant to the development of diabetic nephropathy. Based on the work of these two Projects, we believe that there are shared overall processes between the component projects of the Vanderbilt AMDCC site.

CORES:

The Vanderbilt AMDCC does not have formal Cores as part of its structure. Rather the VU-AMDCC builds on existing infrastructure at Vanderbilt, including the Mouse Metabolic Phenotyping center (MMPC) and the Vanderbilt Ingram Cancer Center, which provide mouse histopathological characterization. Each project has interacted with these cores including the Vanderbilt MMPC and the Vanderbilt small animal imaging core. In addition project 2 closely interacts with the Tennessee Mouse Genome Consortium (TGMC) in order to develop technology to map novel ENU induced mutations identified to confer risk for diabetic nephropathy.

Collaborations with other Groups (Including Core Facilities):

Dr. Breyer has collaborated will all members of the nephropathy group of the AMDCC to write a review on mouse models of diabetic nephropathy which was recently published by JASN. We also continue to collaborate with Dr. Kumar Sharma and Erwin Bottinger in the AMDCC program at Jefferson and Einstein to evaluate the adequacy of HPLC determined serum creatinine as an endogenous marker for determining GFR in conscious mice. These studies should provide a rapid and validated measure of GFR in mice.

We have also shipped eyes from diabetic mice from KK, DBA, C57BL/6 and FVB to Tim Kern for characterization of the severity of their retinopathy. Dr. Kern is also measuring glycated Hb with us to correlate these values with HbA1C values and serum glucose.

Pertinent non-AMDCC Collaborations

The VU AMDCC is collaborating with the VU-MMPC to determine the appropriate measurement for glycated Hbs in mice and comparing these to blood glucose. We are undertaking a pilot project looking at 24 hour continuous glucose monitoring in mice instrumented with a subcutaneous glucose sensor (MiniMed, Germany).

We have also initiated collaborations with Dr. Charles Epstein at Univ of Louisville to examine the FVBOVE26 model of type I diabetes mellitus, Dr. Mary Loeken, at Harvard to study the FVB/N Akita mouse, and Dr. Ambra Pozzi at Vanderbilt, to study the Balb/C-Akita mouse. We have also initiated collaboration with Dr. Rob Williams at Univ Tennessee, Memphis to plan the mapping of Quantitative trait loci that could contribute to DN in the prone DBA/2J mouse vs. the resistant C57BL/6 mouse.

Address previous EAC comments:

- *Using ENU mutation to identify genes causing diabetes is an excellent goal, and this is an important approach to finding new genes. In fact, it is exciting that several potentially new lines were presented. However, toward the goals of this consortium, this great approach needs to be tempered with identification and full characterization of these new strains. One feeling in this entire consortium meeting is that there are so many models yet none are characterized in depth. This project has the greatest potential problem of creating too many models.*

We agree. Certainly, with the available resources, we cannot phenotype all 5 candidate mutant lines. We will primarily focus on ENU76. We are expanding this line and which is presently on generation G3, and will continue to characterize its renal phenotype as well as expand our phenotyping of the retinopathy, nerve, uropathy and atherosclerosis. Unfortunately it is takes quite some time to expand this colony since only 25% of the offspring have both the Akita (type 1 mutation) and the dominant ENU "nephropathy" mutation. Furthermore since it takes a full year to identify significant changes consistent with nephropathy, these mice are still fairly young and full phenotyping cannot be performed.

- *Several questions were raised by Dr. Breyer during the presentation including the time point in which to look for phenotypes and whether phenotypes other than kidney should be examined. The EAC would recommend testing mice by placing on one of the diets used by others, such as the Western diet, and checking function at a time point used by others such as 12 wks. In addition, other simple tests such as plasma lipids and/or lipoprotein levels would be included as mice don't really develop atherosclerosis unless lipids are altered. Thus, potentials for atherosclerosis could be easily screened.*

We agree that this shorter time period of treatment would be helpful for determination of atherosclerosis. This is readily applicable to the study of strain study in the context of diabetes. In contrast, as stated above, the number of mice for each ENU or eNOS-/-db/db genotype is, at present relatively limited, so we'd be loath to sacrifice them before they achieve an age sufficient to exhibit the later stages of nephropathy. However if any of the atherosclerosis centers voice interest in phenotyping these mice for vascular disease, we'd be happy to share the main mutant candidate(s) with that group. At present, our resources preclude us from performing studies specifically examining effects of dietary fat and atherosclerosis locally.

- *The results of the ENU screen are very intriguing and it's good to see the characterization of these mice continued.*

We appreciate the EAC's support in this endeavor and, as stated above are pursuing this characterization.

- *Should be encouraged to map existing random mutants – these are also excellent candidates to send to Core Leaders for phenotyping retinopathy, neuropathy, and uropathy.*

We are studying strain variability, to determine which diabetic strains and F1, F2 intercrosses exhibit the least albuminuria. Once we find a strain that, like C57BL/6 exhibits little albuminuria in the setting of diabetes, we will use this strain to map the

mutation. Our preliminary studies suggest Balb/C will be a good strain for this purpose and we have already begun to back-cross the Akita mutation onto Balb/C (N8).

- *Should continue to take the lead in standardizing methods for measuring HbA1c.*

We are performing studies in collaboration with the MMPC at Vanderbilt. Many methods have been developed for measuring HbA1c including using HPLC and immunoaffinity techniques (1, 2). The DCA 2000 instrument (Bayer Diagnostics, Milan, Italy) was designed to determine HbA1c based on a latex immunoagglutination inhibition methodology using a mouse anti-human HbA1c monoclonal antibody. A correlation between HbA1c values measured using DCA 2000 instrument and Diamat HPLC approach has been demonstrated in humans (1).

We will determine whether HPLC vs. DCA2000 is the preferable method for measuring glycated Hb in mice. In other studies we will correlate glycated Hb/HgA1c with blood glucose levels determined continuously using a subcutaneous glucose sensor implanted into mice. This also should help validate the utility of using fasting blood sugar measurements in mice as well as determine the circadian patterns of glucose regulation in different strains of mice.

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PART B:

UPDATE BY PROJECT LEADERS

Responsible Investigators: **Raymond C. Harris, M.D.**

Project Number and Title: **Project 1 – " Characterization of Candidate genes predisposing to diabetic nephropathy"**

A. Rationale and Relevance:

Modifier genes have been proposed to be important for the development of diabetic nephropathy, the rate of progression of the injury (from microalbuminuria to ESRD) or both. Population studies have identified a number of potential candidate genes that may predispose to progressive nephropathy in either type I or type II diabetes. In this regard, we are concentrating on the possible interaction of altered endothelial function and altered regulation of lipoproteins as exacerbating factors in type II diabetes. Among the candidate loci in people, showing evidence for association with DN, are eNOS and ApoE3 polymorphisms. We therefore examined the effects of deletion of these genes on the development of diabetic nephropathy in mice.

These studies were initiated in a model of type II diabetes mellitus. (i.e. insulin resistance). Mice carrying the *db* mutation of the leptin receptor (i.e. *LepR*^{db}) do not respond to leptin over eat and homozygous *db/db* mice become obese by 3 to 4 weeks of age. Plasma insulin is elevated by 10 to 14 days and blood sugar is elevated by 4 to 8 weeks. It is well known that the course and severity of the disease is influenced by genetic background of the mice. On the C57Bl/6J background, islet ß-cells undergo compensatory hyperplasia, and the mice display continued hyperinsulinemia throughout an 18-to 20-month life span.

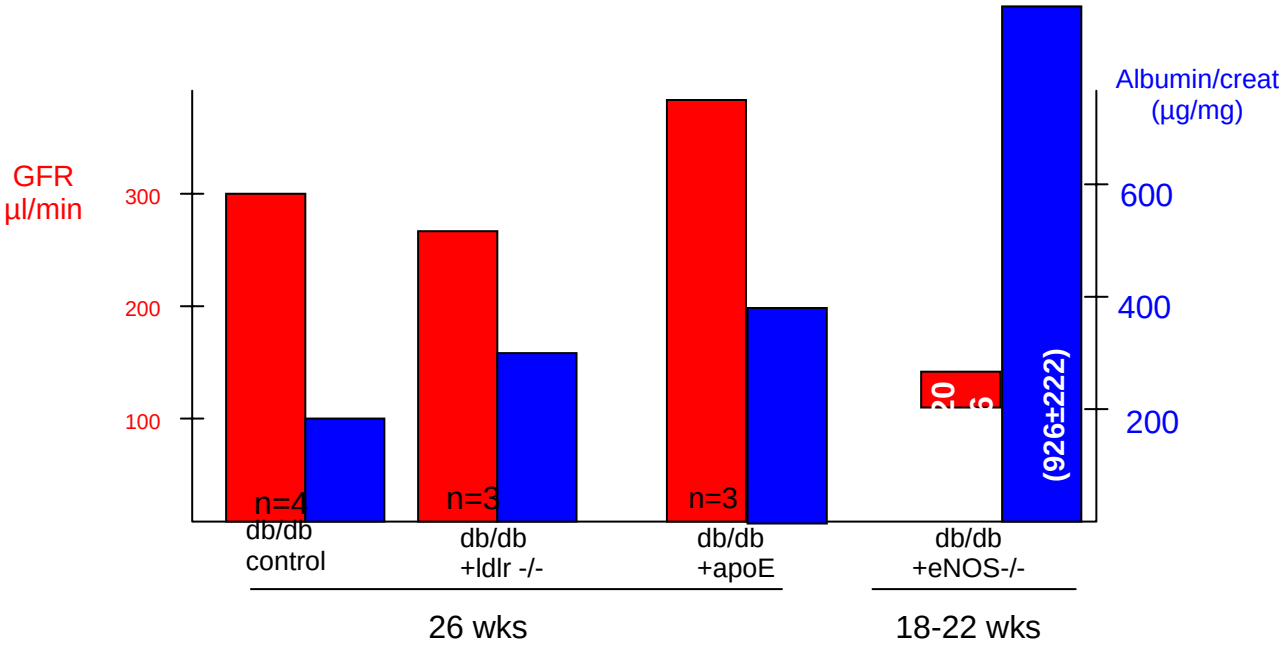
The *db/db* mice on the C57BL/6J background develop minimal nephropathy. In contrast, when *db/db* is expressed on the C57BLKs background, the diabetes is more severe due to an insulinitis that results in progressive depletion of the insulin-producing ß-cells of the pancreatic islets, and death by 10 months of age. This past year we completed the backcross at the backcross of several modifier genes including ApoE-/-, LDLR-/- and eNOS-/- from C57BL/6 to C57BLKS at the 10th generation (N10). In the section below we provide an initial characterization of the nephropathy in these strains.

B. Summary of accomplishments

In 26-week-old non-obese C57BLKS mice (control), albumin to creatinine ratio (ACR) was 94 ±10 mg/g (n=3), higher than in C57BL6/J mice. GFR was 199±3 µl/min/mouse (n=3). Albumin/creatinine ratio at 26 weeks was higher in diabetic C57BLKS *db/db* mice than their non-diabetic littermates, being 143±28 (n=7) GFR was not different 175 (n=10).

In preliminary studies, we examined the N10 backcross (expected to be >99% pure) of three mutations with LepR$^{db/db}$ mice on the C57BLKS background. Of note **26**-week db/db mice with concomitant LDL receptor deletion, had a modestly increased

albumin/creatinine ratio (see blue bars fig 1) while in the **26**-week mice with ApoE gene deletion ACR was more significantly increased to 389µg/mg. However neither of these two hyperlipidemic models exhibited a decline in GFR. In contrast, the eNOS-/- mouse not only exhibits an dramatic increase in albuminuria, but also shows a significant decline in GFR as compared to eNOS wild-type diabetic (db/db C57BLKS mice).



C. Plans for the coming year

We will expand studies of the eNOS-/- mouse to a model of type I diabetes mellitus (Akita and Tg-pdx1HNF6 mice). Other studies will examine the pace of development of nephropathy in heterozygous eNOS-/+ diabetic mice.

D. Significant achievement and its importance

Endothelial nitric oxide synthase (eNOS) is a key enzyme in maintaining blood pressure and normal endothelial function. A polymorphism in eNOS is associated with increased risk of diabetic nephropathy as well as increased rate of development of diabetic nephropathy in people(3-5). These studies suggest that eNOS dysfunction might also predispose to diabetic nephropathy in mice. Since ApoE polymorphisms have been associated with diabetic nephropathy in humans, hypofunctioning mutations could confer the necessary conditions in mice to exacerbate diabetic nephropathy.

Publications

ENOS null x db/db paper in preparation.

Responsible Investigators: **Matthew D. Breyer, M.D.**

Project Number and Title: **Project 2 – " A screen for dominant ENU mutants developing diabetic nephropathy"**

A. Rationale and Relevance:

Evidence suggests that, as in man, genetic modifiers are a critical determinant of the extent of renal injury in mice (6, 7). The extent of renal injury developing in mice with insulin dependent diabetes (e.g. following streptozotocin treatment) is dramatically influenced by the genetic background. Even in "susceptible" mouse strains, it remains unclear whether predisposition to histopathologically defined glomerulosclerosis, is accompanied by diminished glomerular filtration rate (GFR) as is typically observed in humans with DN (8).

In this project we are utilizing *N*-ethyl-*N*-nitrosourea (ENU) to perform a "sensitized screen" for dominant mutants that convert the diabetic nephropathy "resistant" C57BL/6 mouse strain, to a "susceptible" strain that develops glomerulosclerosis, albuminuria and renal failure. ENU is a potent point mutagen that acts by transferring its ethyl group to oxygen or nitrogen radicals in DNA, which, if not corrected, results in mis-pairing with resulting A/T to T/A transversions and A/T to G/C transitions (9). ENU mutagenesis provides an unbiased approach for identifying novel and unpredicted genes that may contribute to the development of DN throughout the genome.

B. Summary of Accomplishments

GENERATING novel Mouse mutants with diabetic nephropathy:

During the prior year of support, we identified four type I diabetic Akita C57BL/6J ENU mutants exhibiting increased albuminuria. These mutants exhibiting increased albuminuria with a Ualb: UCreat ratio averaging 110±50 µg/mg. This value is more than 3 standard deviations more than the typical levels of albuminuria in C57BL/6J^{ins2akita}

Table 1: serum Creat [HPLC] and urine ACR in four mutant lines with and without type 1 diabetes [akita mutation].

ENU founders line (G1)	Total # of G2 mice	Total # of G3 mice	# of mice phenotyped	Creat mg/dl	ACR (ug/mg)	
					Akita -	albuminuric Akita+
ENU10	6	currently breeding	6	0.141 ±0.02	36.0 ±9.3 n=2	**98.0 ±3.6** n=2
ENU76	28	38	50	0.180 ±0.01 p<0.005	19.5 ±7.7 n=16	**125.4 ±6.2** n=3
ENU20	20	51	70	0.090 ±0.02	11.8 ±7.7 n=11	**108.8 ±44.5** n=9
ENU161	6	currently breeding	6	0.07	22.3 ±6.4 n=2	**110.1 ±9.0** n=2

mice, defining them as prime candidates for founders carrying novel mutations conferring susceptibility to diabetic nephropathy.

We have now confirmed that this increased albuminuria is heritable in these four lines. Equally important is that two of these lines exhibit increased serum creatinine as measured by HPLC and more dramatic glomerulosclerosis (see table I preceding page).

C. Plans for the coming year

The top priority for the upcoming year will be to complete the phenotyping of the primary candidate ENU line 76. This will be accomplished by continuing to segregate the dominant mutation away from other extraneous ENU mutations in this line. The severity of renal insufficiency will be determined by measuring GFR at later stages of life (12-16 months) as will histopathology. We will also start mapping the mutation by establishing the Akita mutants on Balb/C and intercrossing Balb/C with the ENU mutant line 76.

We hope to complete a manuscript describing the severity of nephropathy in the C57BL/6J Akita mouse as well as continue backcrossing the Akita mutation to Balb/C and DBA2J strains.

D. Most significant achievement.

The identification of four diabetic ENU induced mutants that exhibit progressively worsening albuminuria, significantly exceeding that detected in other diabetic siblings. In particular ENU 76 exhibits increased creatinine and severe renal histopathology. We are optimistic that this may provide another model of diabetic nephropathy.

Publications

Manuscript on diabetic nephropathy in Akita mouse in preparation

Responsible Investigators: **Matthew Breyer M.D. and Agnes Fogo M.D.,**

Project Number and Title: **Project 3 – " Phenotypic Screens for diabetic nephropathy in inbred strains of mice"**

A. Rationale and relevance

1. *Phenotyping diabete and renal function in mice:*

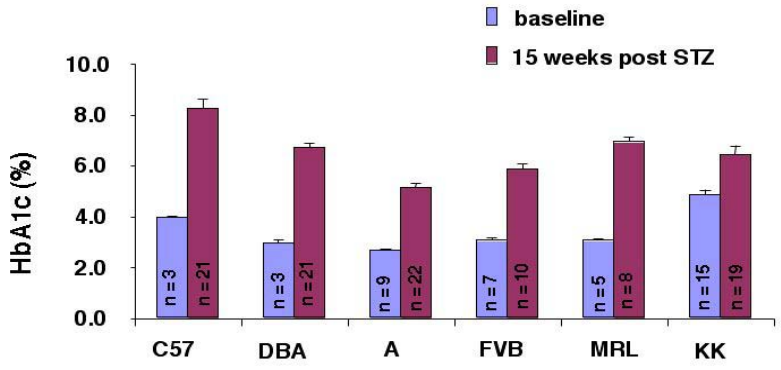
Fasting blood sugar and glycated hemoglobin are two key parameters that correlate with the development of diabetic complications in people. While we assume the same will hold true in mice, we don't know that this is the case. Furthermore, we haven't validated the technique of measuring FBS and glycated hemoglobin in mice or determine the variability in these measurements by strain of diabetic mice.

The ability to identify renal disease in mice has been significantly hampered by the lack of the routine and easy methods to determine GFR using inulin clearance and/or serum creatinine.

The validation of urine albumin to creatinine ratio as compared to daily urine albumin excretion has not been established for diabetic mice. Furthermore the normal levels of albuminuria in commonly used mouse strains have not been established.

B. Summary of Accomplishments:

We have measured HbA1c levels in six inbred mice with hyperglycemia induced by streptozotocin injection using the immunoaffinity based Bayer DCA 2000 instrument. The results indicated a significant correlation between the levels of HbA1c and fasting blood glucose in C57BL/6J and KK/HlJ mice, but not in other studied strains including DBA/2J, A/J, MRL/MpJ, and FVB/NJ mice. We also found the HbA1c values measured using DCA 2000 were relatively lower than in humans. Finally preliminary studies suggest that HPLC determination of glycated Hb may be more sensitive to changes in blood glucose inmice than the immunoaffinity-based technique.

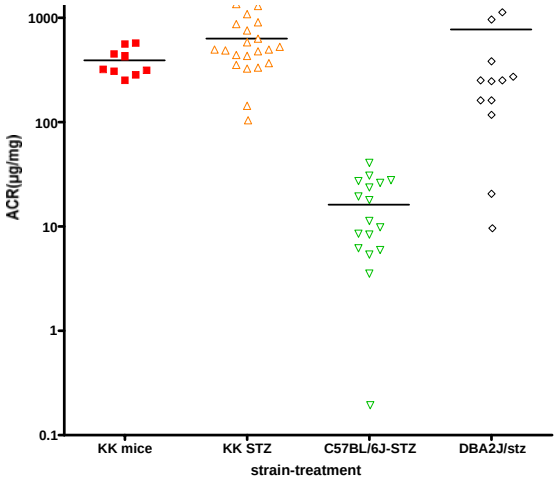


In the past three year we have established the feasibility of serially measuring GFR in conscious mice using FITC-inulin clearance(10). We have also established the HPLC

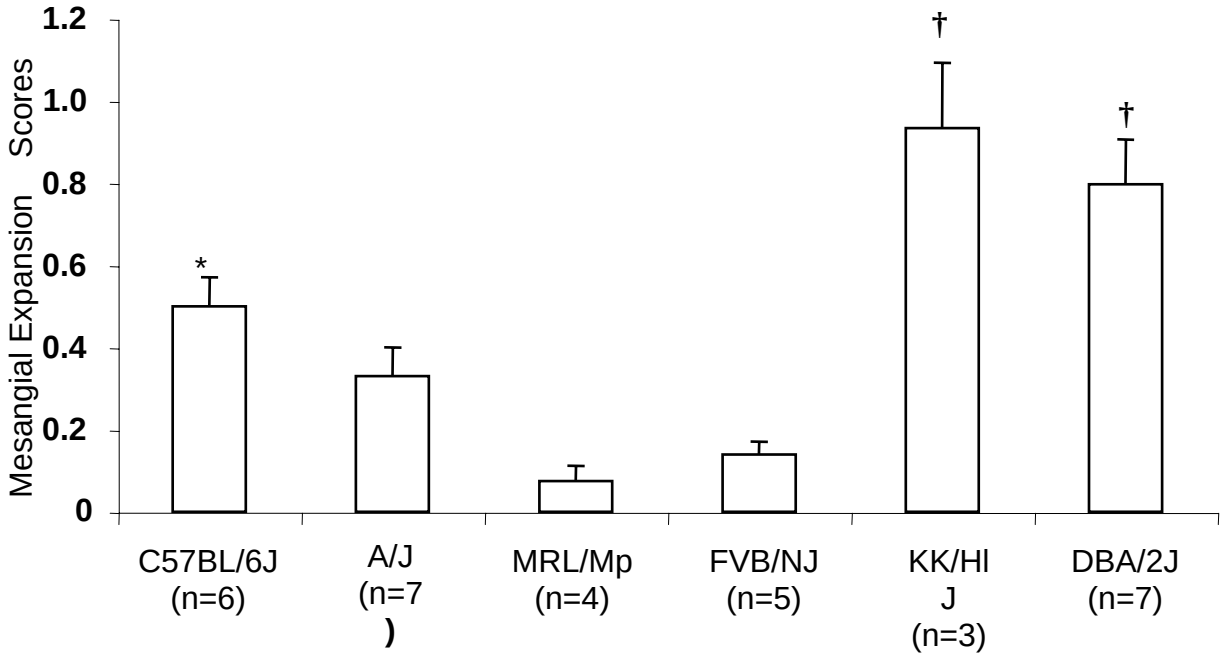
measured plasma creatinine and creatinine clearance roughly correlate with renal function and steady-state measures of FITC inulin clearance (11).

Studies examining the levels of albuminuria in several strains of inbred diabetic mice have been performed. In these studies we've demonstrated that DBA2/J and KK/HiJ develop significantly more albuminuria than in diabetic C57BL6/J mice of comparable age, gender, duration and severity of diabetes (induced by low dose STZ). Interestingly non-STZ treated KK mice also exhibit substantial albuminuria as compared to diabetic C57BL6/J mice (Figure 3).

Figure 3: Log10 plot of Alb:Cre ratio in diabetic KK, C57BL/6J and DBA2/J mice. Measurements were made following 15 weeks of hyperglycemia in male mice. Symbols represent ACR in individual mice.



Importantly the severity of albuminuria correlates with the degree of mesangial sclerosis as determined by semi-quantitative scoring of perfuse-fixed kidney sections (see figure 4 below).
We have submitted a manuscript for publication on the strain variability of diabetic nephropathy.



C. Plans for the coming year

We will correlate non-steady state GFR measurements in diabetic and non-diabetic mice with serum HPLC creatinine. The hope would be to establish an equivalent formula for mice as “cockcroft gault” (used in humans) so that inulin clearance can be estimated from measured HPLC plasma [creatinine].

A pilot study looking at an F2 intercross between C57Bl6 and DBA2 has been undertaken to determine the feasibility of performing QTL mapping of DN genes in these two strains.

The Akita allele has been backcrossed to DBA2, FVB, and BalbC. The course of diabetic nephropathy in these models of type I diabetes mellitus will be studied.

Collaborations between the Einstein/Jefferson Group and the Vanderbilt Group will be undertaken to utilize microarray to identify specific genes that are up-regulated in kidneys of diabetic strains exhibiting greater susceptibility to nephropathy than those resistant to nephropathy.

D. Significant Achievement

Establishing strain dependence of diabetic nephropathy as determined by albuminuria and histopathological changes. The studies suggest KK/HiJ and DBA2/J mice provide good candidate strains for further exploration as to whether they may develop diabetic nephropathy including renal failure.

Publications:

Zhonghua Qi[1], Hiroki Fujita[1], Jianping Jin Linda S. Davis Agnes B. Fogo Matthew D. Breyer Identification of Inbred Mouse Strains Susceptible to Diabetic Nephropathy (submitted).

M. D. Breyer, Erwin Böttinger, Frank C. Brosius, III, Thomas M. Coffman, Raymond C. Harris, Charles W. Heilig, and Kumar Sharma (For the AMDCC) Mouse Models of Diabetic Nephropathy. J Am Soc Nephrol. 2005 Jan;16(1):27-45.

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